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Leishmania (*Viannia*) *braziliensis* nucleoside triphosphate diphosphohydrolase (NTPDase 1): Localization and *in vitro* inhibition of promastigotes growth by polyclonal antibodies

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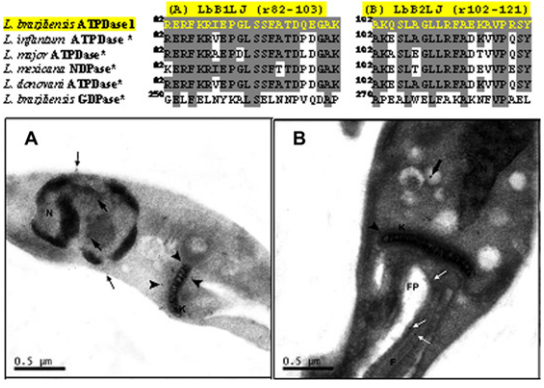
HIGHLIGHTS

- Antigenic domain was identified within NTPDase 1 from *Leishmania (Viannia) braziliensis*.
- Anti-peptides antibodies were used as tools for studies of this isoform.
- It was immunolocalized on the surface and subcellular sites.
- The NTPDase 1 activity was inhibited by immune sera anti-peptides.
- Immune sera anti-peptides significantly reduce *in vitro* promastigotes growth.

GRAPHICAL ABSTRACT



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ABSTRACT

Nucleoside triphosphate diphosphohydrolase (NTPDase) activity was recently characterized in *Leishmania* (*Viannia*) *braziliensis* promastigotes (Lb), and an antigenic conserved domain (r82–121) from the specific NTPDase 1 isoform was identified. In this work, mouse polyclonal antibodies produced against two synthetic peptides derived from this domain (LbB1LJ, r82–103; LbB2LJ, r102–121) were used. The anti-LbB1LJ or anti-LbB2LJ antibodies were immobilized on protein A-sepharose and immunoprecipitated the NTPDase 1 of 48 kDa and depleted approximately 40% of the phosphohydrolytic activity from detergent-homogenized Lb preparation. Ultrastructural immunocytochemical microscopy identified the NTPDase 1 on the parasite surface and in its subcellular cytoplasmic vesicles, mitochondria, kinetoplast and nucleus. The ATPase and ADPase activities of detergent-homogenized Lb preparation were partially inhibited by anti-LbB1LJ antibody (43–79%), which was more effective than that inhibition (18–47%) by anti-LbB2LJ antibody. In addition, the immune serum anti-LbB1LJ (67%) or anti-LbB2LJ (33%) was cytotoxic, significantly reducing the promastigotes growth *in vitro*. The results appoint the conserved domain from the *L. braziliensis* NTPDase as an

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important target for inhibitor design and the potential application of these biomolecules in experimental protocols of disease control.

1. Introduction

Leishmania (*Viannia*) *braziliensis* is a protozoan parasite transmitted by the bite of female phlebotomine sandflies. It is found throughout Brazil and causes American cutaneous leishmaniasis, a disease characterized by the development of single or multiple cutaneous lesions (Marques et al., 2006; Passos et al., 1999). The structural organization and metabolism of *Leishmania* spp. have been studied and several proteins have been indicated as potential targets of antileishmanial drugs (Costa et al., 2011; Cuervo et al., 2009; De Souza, 2008; Silverman et al., 2008).

The nucleoside triphosphate diphosphohydrolase (NTPDase; EC 3.6.1.5), which is also named apyrase in plants, and ATP diphosphohydrolase, NTPDase or NTPase in parasites, hydrolyzes di- and triphosphate nucleosides to the corresponding nucleoside monophosphates upon bivalent metal ion activation. These ubiquitous enzymes are insensitive to the F-, P- and V-type ATPases inhibitors and share five apyrase-conserved regions (ACR1–ACR5) that are involved in the catalytic cycle (Coimbra et al., 2002; Faria-Pinto et al., 2004; Guevara-Flores et al., 2008; Kettlun et al., 2005; Knowles, 2011; Ruckert et al., 2010; Sansom, 2012). Biological actions of NTPDases are related to the regulation of nucleotides, and are involved in several physiological processes such as purinergic signaling systems, and infectivity and/or purine recuperation by protozoan parasites, and these proteins have been appointed as possible targets for the treatment of several diseases (Burnstock and Verkhratsky, 2009; Gendron et al., 2002; Kikuchi et al., 2001; Meyer-Fernandes et al., 2010; Paletta-Silva and Meyer-Fernandes, 2012; Penido et al., 2007; Pinheiro et al., 2006; Sansom, 2012; Santos et al., 2009; Souza et al., 2010; Tan et al., 2010).

Recently, an active and antigenic NTPDase isoform, the NTPDase 1, was characterized in *L. braziliensis* promastigotes (Rezende-Soares et al., 2010), the first stage of host–parasite interaction that includes modulation of the immune response and host–cell signaling (Cuervo et al., 2009; De Souza, 2008). By experimental and *in silico* analyses we identify a particular conserved domain, named B, within *L. braziliensis* NTPDase 1, which is also shared with potato apyrase and NTPDases of other plants and pathogenic organisms of distinct phylogenetic lineages. The domain B did not include amino acids sequences of ACRs, and it was associated to antigenicity in human parasite diseases, and also seems to be conserved during host and parasites co-evolution (Coimbra et al., 2008; Faria-Pinto et al., 2004, 2006, 2008, 2010a,b Maia et al., 2011; Mendes et al., 2011; Rezende-Soares et al., 2010; Vasconcelos et al., 2009).

In preliminary analysis, the localization of NTPDase activity in *L. braziliensis* promastigote forms was obtained by ultrastructural cytochemical (Rezende-Soares et al., 2010), but this technique did not distinguish the NTPDase 1 and a putative GDPase (Peacock et al., 2007; XP_001562788), also annotated in the genome of this parasite. In this work, mouse polyclonal antibodies against two synthetic peptides (LbB1LJ, r82–103; LbB2LJ, r102–121) belonging to the domain B from the *L. braziliensis* NTPDase 1 (Peacock et al., 2007; XP_001562178) were used for specific identification and localization of this protein in promastigote forms. In addition, the effects of mouse polyclonal anti-peptide antibodies on the NTPDase 1 activity, using a suitable reaction medium for both biochemical and immunological assays, and *in vitro* proliferation of *L. braziliensis* promastigotes were tested.

2. Material and methods

2.1. Potato apyrase, synthetic peptides and development of polyclonal antibodies

Potato apyrase was purified from a commercial strain of *Solanum tuberosum* and used to obtain polyclonal antiserum in a New Zealand white rabbit (Faria-Pinto et al., 2004; Kettlun et al., 2005). The LbB1LJ (r82–103; Fig. 1B; Rezende-Soares et al., 2010) and LbB2LJ (r102–121, Fig. 1B) belonging to a conserved domain from *L. braziliensis* NTPDase 1 (Peacock et al., 2007; XP_001562178.1; 47,689 Da) were obtained by solid-phase peptide synthesis as earlier described (Korkmaz et al., 2008). The molecular mass and purity of synthesized peptides were confirmed by amino acid analysis and

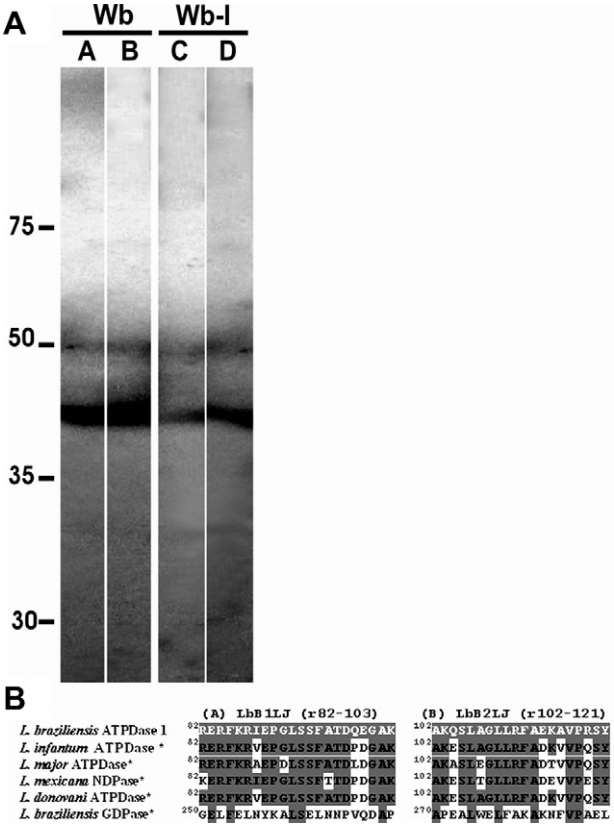

Fig. 1. (A) Reactivity of active NTPDase 1 from *L. braziliensis* promastigote with polyclonal anti-peptides antibodies. An aliquot (100 μg of protein) of *L. braziliensis* promastigote preparation (Wb, A and B) was submitted to electrophoresis in 10% SDS–PAGE, electroblotted onto nitrocellulose membrane, and the Western blots was developed with mouse polyclonal serum (dil. 1:200) against LbB1LJ (A) or LbB2LJ (B). In Wb-I, immunoprecipitated protein A-mouse antibody–antigen complex isolated from promastigote preparation by mouse polyclonal anti-LbB1LJ (C) or anti-LbB2LJ (D) antibodies was electrophorezed and electroblotted under same conditions, and developed with rabbit polyclonal serum against potato apyrase (dil. 1:1000). The membranes were revealed by chemiluminescence. (B) Alignment of primary amino acid sequence of the synthetic peptide LbB1LJ (A) or LbB2LJ (B) and its counterpart within putative proteins from *Leishmania* spp.*. The identical amino acid residues of the peptides LbB1LJ and LbB2LJ are shown as grey columns. GenBank accession numbers of the amino acid sequences are: *L. braziliensis* ATPDase 1, *XP_001562178, *L. infantum* ATPDase, CAM66723.1; *L. major* NDPase, CAJ03227.1; *L. mexicana* NDPase, CBZ25018.1; *L. donovani* ATPDase, CBZ32820.1; *L. braziliensis* GDPase, XP_001562788.

by MALDI-TOF using a Microflex – LT mass spectrometer (Bruker–Daltonics, Billerica, MA, USA). These peptides span the domain B (r82–121) within *L. braziliensis* NTPDase 1 overlapping two amino acids at LbB1LJ C-terminus and at LbB2LJ N-terminus. They maintain the predicted epitopes available for antibody binding (Faria-Pinto et al., 2008; Vasconcelos et al., 2009).

Polyclonal immune serum against each peptide (10 μg) was obtained from seven-week old BALB/c mice that were inoculated by a peritoneal route emulsified in Freund's complete and incomplete adjuvant, delivered in 15-day interval as early described (Mendes et al., 2011). In addition, pooled serum samples obtained from BALB/c mice that were inoculated under the same experimental conditions with both complete and incomplete Freund adjuvant in the absence of the peptide were used for specificity control in all experiments (Mendes et al., 2011). The sera were stored at −20 °C. The study protocols complied with the regulations of the Brazilian National Council of Research in Animals and were approved by the Ethical Committee for Animal Research of the Universidade Federal de Juiz de Fora, Juiz de Fora, MG, Brazil, under process nº.012/2011.

2.2. Identification of active NTPDase 1 by immunoprecipitation assays

Promastigote preparations from *L. (V.) braziliensis* MHOM/BR/1975/M2903 strain were obtained as described elsewhere (Rezende-Soares et al., 2010). An aliquot of promastigotes preparation was suspended in standard reaction medium containing 50 mM MOPS (3-(*N*-morpholino) propanesulfonic acid) buffer, pH 7.4, 1 mM $CaCl_2$, 100 μM sodium orthovanadate supplemented with 1 mg/ml dodecyl nonaethylene glycol ether (C12E9). After centrifugation at 10,000*g* for 10 min at 4 °C, mouse immune serum anti-LbB1LJ or anti-LbB2LJ at a final dilution of 1:200 was added to the aliquots of high-speed supernatant (5 mg protein/ml) and incubated for 3 h at room temperature. Assays using control serum were run in parallel. Protein A-Sepharose was added and incubated for an additional 2 h. Supernatants were used for phosphohydrolytic activity measurements in triplicate in standard reaction medium containing 0.1 mg protein/ml. The reaction was initiated by addition of 3 mM ATP or ADP, allowed to proceed for 60 min at 37 °C, and the amount of inorganic phosphate (Pi) liberated was determined spectrophotometrically (Rezende-Soares et al., 2010). The experiments were repeated twice. The resin-mouse antibody–antigen complexes were sedimented by centrifugation for 5 min, washed 3 times in 50 mM MOPS buffer, pH 7.4, solubilized in gel loading buffer and submitted to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS–PAGE), using Mini-Protean III Cell (Bio-Rad). The proteins were electroblotted onto nitrocellulose membranes, followed by blocking step (0.15 M phosphate buffer solution, pH 7.4, plus 0.3% Tween-20 and 2% casein) using standard procedures (Rezende-Soares et al., 2010). To avoid reactivity with subunits from mouse-IgG, rabbit immune serum (diluted 1:1000) containing polyclonal anti-potato apyrase antibodies, diluted in the same blocking buffer without Tween-20, were incubated overnight. Total proteins (100 μg) of *L. braziliensis* promastigote preparations were also submitted to electrophoresis in 10% SDS–PAGE and electroblotted onto nitrocellulose membranes, and developed with immune serum anti-LbB1LJ or anti-LbB2LJ (diluted 1:200). Signals were revealed by chemiluminescence using anti-rabbit IgG or anti-mouse IgG antibody coupled to horseradish peroxidase and luminol as substrate (ECL Western blotting system; GE Healthcare, Brazil) and exposed to X-ray film following the manufacturer's instructions.

2.3. Effects of mouse polyclonal anti-peptide antibodies on the phosphohydrolytic activity

Aliquot of promastigotes preparation was homogenized in C12E9, centrifuged, and the supernatant was incubated for 3 h with serum containing mouse polyclonal anti-peptide antibodies or mouse control serum at a final dilution of 1:200 exactly as described in Section 2.2. Aliquots were used for phosphohydrolytic activity measurements in triplicate in standard reaction medium. The assays were repeated in three different experiments.

2.4. Ultrastructural immunocytochemistry

For analysis by transmission electron microscopy, the promastigotes were fixed in freshly prepared 4% paraformaldehyde/0.1% glutaraldehyde and 0.2% picric acid in a 0.1 M sodium cacodylate buffer, pH 7.2. Samples were dehydrated in methanol at progressively lower temperatures, embedded in Lowicryl K4 M resin (Polysciences, Inc., PA, USA) and maintained at –20 °C. Ultrathin sections were collected on 300 mesh nickel grids and incubated for 30 min at 25 °C in 50 mM ammonium chloride in 0.1 M PBS, pH 8.0. Afterward, sections were incubated for 10 min at room temperature in PBS containing 3% BSA and 0.2% Tween 20 (PBS–BSA–TW), pH 8.0 and then incubated overnight in the presence of immune serum anti-LbB1LJ, anti-LbB2LJ or control serum diluted 1:100. Grids were rinsed in PBS–BSA and, finally, incubated for 60 min with a 1:20 dilution of goat anti-mouse antibody conjugated with 10 nm gold particles (Sigma Chemical Co., St. Louis, MO, USA). The grids were subsequently washed with PBS and distilled water, stained with uranyl acetate and observed in a Jeol 1011 transmission electron microscope from Electronic Microscopy Platform of the Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, RJ, Brazil.

2.5. In vitro antileishmanial activity

The antileishmanial activity of the polyclonal anti-LbB1LJ or anti-LbB2LJ antibodies was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method based on tetrazolium salt reduction by mitochondrial dehydrogenases, as earlier described (Coimbra et al., 2010). Briefly, mouse polyclonal anti-peptide antibodies at a final dilution of 1:100 were incubated for 24 h with promastigotes from a logarithmic phase growth. Controls containing medium alone, or serum samples (diluted 1:100) from either healthy rabbit or BALB/c mice pre-inoculated with complete and incomplete Freund adjuvant in the absence of the peptide (mouse control serum) were also included in this assay. All serum samples were previously incubated at 56 °C for 30 min for the complete inactivation of the complement system. Absorbance was measured at 570 nm (Multiskan MS microplate reader, LabSystems Oy, Helsink, Finland). Amphotericin B (Cristália, São Paulo, Brazil) was used as a control. The analysis was made in triplicate in three separate experiments, and the results represent the mean ± standard deviation. Data were analyzed statistically by the Student's *t*-test with the level of significance set at $P < 0.05$.

3. Results

3.1. Identification of active NTPDase 1 isoform by immunoprecipitation assays

In Western blots of *L. braziliensis* promastigote preparation (Fig. 1A), mouse polyclonal anti-LbB1LJ (Wb, lane A) or anti-LbB2LJ (Wb, lane B) antibodies recognized bands of approximately 48 and 43 kDa. Mouse control serum diluted 1:200 did not react with this preparation (data not shown). In addition, under employed experimental conditions, mouse polyclonal anti-LbB1LJ antibodies immobilized on Protein A-Sepharose immunoprecipitated approximately 30% of the ATPase and 40% of the ADPase activity whereas

anti-LbB2LJ immunoprecipitated 53% of the ATPase and 44% of the ADPase activity from the *L. braziliensis* promastigote preparations (Table 1). The immunoprecipitated resin-mouse antibody–antigen complexes were washed and subjected to electrophoresis and Western blots. As observed in Fig. 1A, the mouse polyclonal anti-LbB1LJ or anti-LbB2LJ antibodies immobilized on Protein A-Sepharose immunoprecipitated the same bands of approximately 48 and 43 kDa from the promastigote preparation (Wb-I, lanes C and D), which were recognized by the rabbit polyclonal anti-potato apyrase antibodies (Fig. 1A).

The primary amino acid sequence of the synthetic peptide LbB1LJ (A) or LbB2LJ (B) that belongs to the conserved domain B from *Leishmania braziliensis* NTPDase 1 was aligned with its counterpart within putative proteins from *Leishmania* spp. (Fig. 1B).

3.2. Immunolocalization of the NTPDase 1 from L. braziliensis promastigotes

Localization of the NTPDase 1 was obtained by ultrastructural immunocytochemical techniques using either mouse polyclonal anti-LbB1LJ or anti-LbB2LJ antibodies and a colloidal gold-conjugated secondary antibody (Fig. 2). The gold immunocomplexes of anti-LbB1LJ (A–D) and anti-LbB2LJ antibodies (E–F) appeared on the outer surface of the plasma membrane, at cytoplasmic vesicles, flagellar pocket (FP), flagellum (F), mitochondria (M), kinetoplast (K) and nucleus (N). Promastigote forms incubated with mouse control serum showed no gold particle labeling (data not shown).

3.3. Mouse polyclonal anti-peptide antibodies inhibit the NTPDase 1 activity from L. braziliensis promastigotes preparation

The effect of mouse polyclonal anti-peptide antibodies binding on phosphohydrolytic activity from the *L. braziliensis* NTPDase 1 was tested using the detergent-homogenized preparations and a standard reaction medium (Table 2). Under experimental conditions employed, polyclonal antibodies against LbB1LJ significantly inhibited ($P < 0.01$) the ATPase (79%) or ADPase (43%) activity of promastigotes preparation when compared to the control (Table 2).

Polyclonal antibodies against LbB2LJ also significantly inhibited ($P < 0.05$) the ATPase activity (47%) of promastigotes preparation when compared to the control and, to a lesser extent, the ADPase activity (Table 2; 18%).

3.4. Antileishmanial activity

The effects of the mouse polyclonal anti-peptides on the *in vitro* proliferation of *L. braziliensis* promastigotes were tested (Fig. 3). The promastigotes growth was significantly inhibited in the presence of anti-LbB1LJ (67%; $P < 0.01$) or anti-LbB2LJ (33%; $P < 0.05$) antibodies when compared to the mouse control serum, which represents 100% cell growth (Fig. 3; C3). The promastigotes growth in medium alone (C1), or with serum samples from either healthy rabbit (C2) or mouse control serum (C3) were similar among them (Fig. 3).

Table 1
Depletion of NTPDase 1 activity from detergent-homogenized promastigotes preparation by mouse polyclonal anti-peptides antibodies immobilized on Protein A-Sepharose.

Experimental conditions[a]	ATPase activity[b] nmol Pi mg^{-1} min^{-1}	ADPase activity[b] nmol Pi mg^{-1} min^{-1}
Control	18.7 ± 1.7	6.6 ± 2
Immune serum anti-LbB1LJ	13.2 ± 0.7 (70)	3.9 ± 0.1 (60)
Immune serum anti-LbB2LJ	8.8 ± 0.2 (47)	3.7 ± 1.1 (56)

[a] Mouse control serum or immune serum diluted 1:200.
[b] The result is the mean ± standard deviation of two different experiments made in triplicate. In parenthesis, percentage of hydrolytic activity compared to the control.

4. Discussion

In this work we showed that mouse polyclonal antibodies produced against synthetic peptides LbB1LJ (r82–103) and LbB2LJ (r102–121), which span the antigenic and conserved domain B within *L. braziliensis* NTPDase 1, identified two bands of approximately 48 and 43 kDa in Western blots of both total homogenized and immunoprecipitated resin-mouse antibody–antigen complexes. These polyclonal antibodies immobilized on Protein A-Sepharose immunodepleted approximately 40% of the NTPDase activity from the *L. braziliensis* promastigotes preparation, remaining in the soluble fraction either NTPDase 1 insufficiently depleted or enzyme activity from the putative GDPase (MW 75 kDa) from *L. braziliensis* genome (Peacock et al., 2007), this last one still not characterized in this parasite. It is interesting to observe that no significant identity was found between the synthetic peptide LbB1LJ or LbB2LJ and its counterpart within putative *L. braziliensis* GDPase (Fig. 1B). The results confirmed the previous identification of the NTPDase 1, obtained by the cross-immunoreactivity with potato apyrase (Rezende-Soares et al., 2010) or r-potDomain B, a recombinant polypeptide obtained as a 6xHis tag polypeptide belonging to the domain B (r78–117) from the potato apyrase (Maia et al., 2011). The band of 43 kDa possibly resulted of *in vivo* proteolytic posttranslational processing of the *L. braziliensis* NTPDase 1, as previously observed (Maia et al., 2011; Rezende-Soares et al., 2010). Taken together, these results showed the specificity, sensitivity and affinity of mouse polyclonal anti-LbB1LJ or anti-LbB2LJ antibodies for the epitopes within amino acids residues r82–121 from the *L. braziliensis* NTPDase 1, an important target for inhibitor design. The domain B is highly conserved within putative NTPDases of *Leishmania infantum* and *Leishmania major* (Peacock et al., 2007), as shown by Maia et al. (2011) and, also, of *Leishmania donovani* and *Leishmania mexicana*, recently annotated in the respective genomes (Fig. 1B). Thus, the results suggest a potential application of these antibodies for the study of the structure and function of this protein and, also, of other parasite NTPDase isoforms that share the domain B (Maia et al., 2011).

Parasite NTPDase isoforms have been described by us and by other authors as ecto-enzymes on the parasite surface (Coimbra et al., 2002; Faria-Pinto et al., 2004, 2006; Guevara-Flores et al., 2008; Kikuchi et al., 2001; Meyer-Fernandes et al., 2010; Pinheiro et al., 2006; Ruckert et al., 2010; Sansom, 2012; Santos et al., 2009). Now, by immunocytochemical techniques, mouse polyclonal anti-LbB1LJ or anti-LbB2LJ antibodies specifically identified promastigote NTPDase 1 at the surface of the plasma membrane, in flagellar pocket and flagellum, mitochondria and kinetoplast, in according to the cytochemical method (Rezende-Soares et al., 2010) and, also, in nucleus and associated with cytoplasmic vesicles. The parasite use environment metabolites as energy source for its growth and morphological changes during its life cycle. It is by means of the cell surface that the parasite interacts with its host, either by the direct participation of surface-associated proteins or by secretion of proteins, being the flagellar pocket an organelle with intense endocytic and exocytic activities in *Leishmania* (Costa et al., 2011; Cuervo et al., 2009; De Souza, 2008; Silverman et al., 2008). This ubiquitous distribution of NTPDase 1 in promastigotes suggests its participation in several metabolic pathways potentially involved in nucleotides modulation in early stages of *Leishmania* infection.

The ATPase (79%) and ADPase (43%) activities of detergent-homogenized *L. braziliensis* preparation were partially inhibited by anti-LbB1LJ antibody, which was more effective than that inhibition of ATP (47%) or ADP (18%) hydrolysis by anti-LbB2LJ antibody

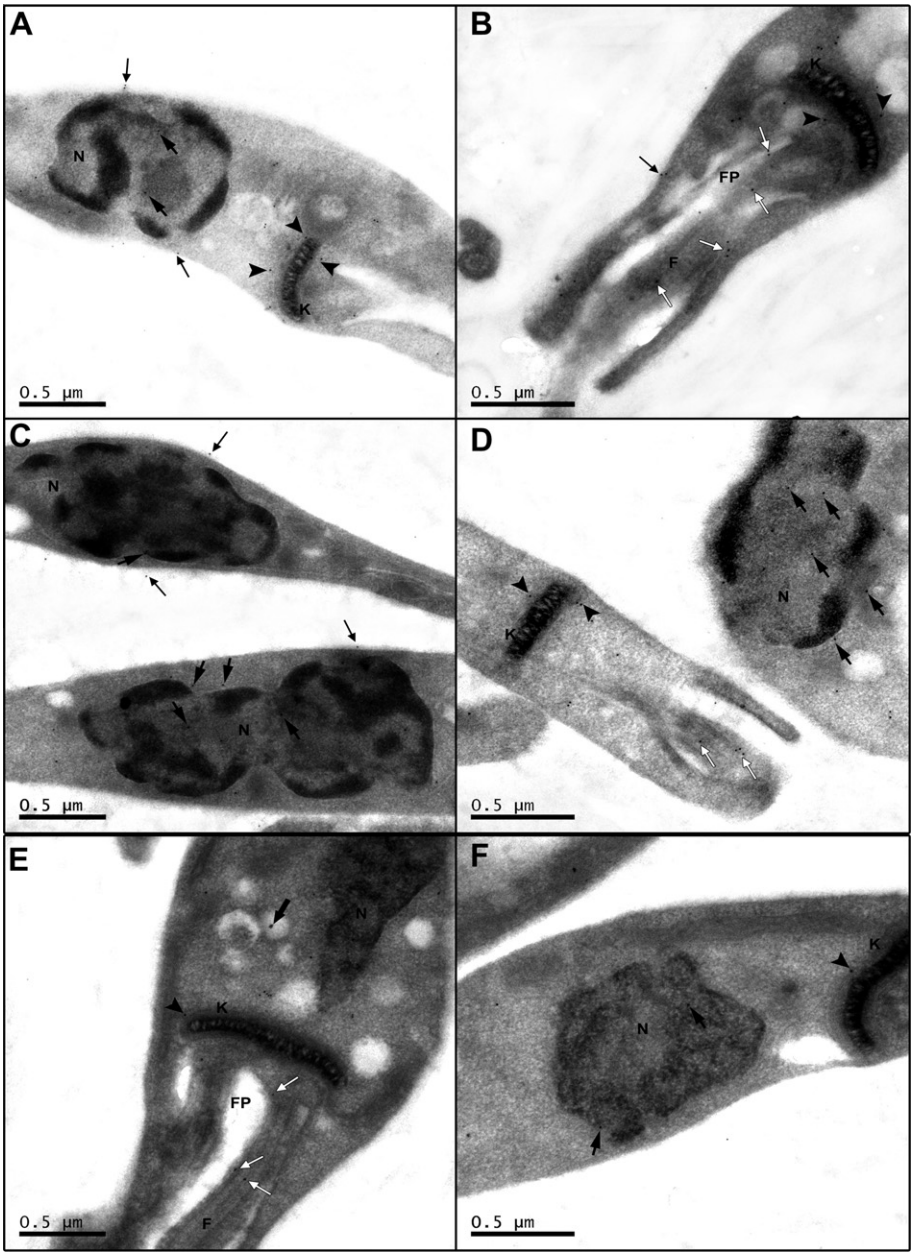

Fig. 2. Electron micrographs showing the localization of NTPDase 1 in *L. braziliensis* promastigotes using immune serum anti-LbB1LJ (A–D) or anti-LbB2LJ (E–F) and a colloidal gold-conjugated secondary antibody. Gold immunocomplexes of anti-peptide antibody are distributed (A, D) at the outer surface of plasma membrane (arrow), nucleus (arrow), mitochondria and kinetoplast (arrowheads); (B) at flagellar pocket and flagellum (white arrows); (C) nucleus and nucleus in division (arrows). Using anti-LbB2LJ antibody, the NTPDase 1 was identified in the same sites, and gold immunocomplexes are shown (E, F) at mitochondria and kinetoplast (arrowheads); flagellar pocket and flagellum (white arrows), cytoplasmic vesicles (arrow) and nucleus (arrow). FL: flagellum; FP: flagellar pocket; K: kinetoplast; N: nucleus. Bar = 0.05 µm.

Table 2
NTPDase 1 activity inhibition by polyclonal anti-LbB1LJ or anti-LbB2LJ antibodies.

Experimental conditions[a]	ATPase activity[b] nmol Pi mg^{-1} min^{-1}	ADPase activity[b] nmol Pi mg^{-1} min^{-1}
Control	17.9 ± 2.7	8.0 ± 2.0
Immune serum anti-LbB1LJ	3.8 ± 2.4 (21)**	4.6 ± 1.3 (57)**
Immune serum anti-LbB2LJ	9.5 ± 0.4 (53)*	6.6 ± 1.4 (82)

In parenthesis, percentage of hydrolytic activity compared to the control.
[a] Mouse control serum or immune serum diluted 1:200.
[b] Result is the mean ± standard deviation of three different experiments made in triplicate.
* *P* value is 0.05.
** *P* value is 0.01.

binding. Possibly, the essential epitope involved with catalytic properties is within r82–103 from *L. braziliensis* NTPDase 1. The significant ATPase activity inhibition by anti-LbB2LJ antibody that binds r102–121, and its lower efficiency for ADPase activity inhibition, appears to indicate that the domain B of the NTPDase 1 participates in nucleotides modulation, again appointing it as an important target for inhibitor design. Since antibodies could has site for calcium, and high molar concentration (1–10 mM) of this bivalent ion could inhibit antibody–antigen binding (Beck et al., 2007; Zhou et al., 2005), we established a suitable standard reaction medium for both immunological and biochemical assays that was useful for isolation of *L. braziliensis* NTPDase 1 from promastigotes preparation by immunoprecipitation assays, as shown here and in previous works (Maia et al., 2011; Rezende-Soares et al., 2010). Possibly this reaction medium maintains the NTPDase 1 closer to its

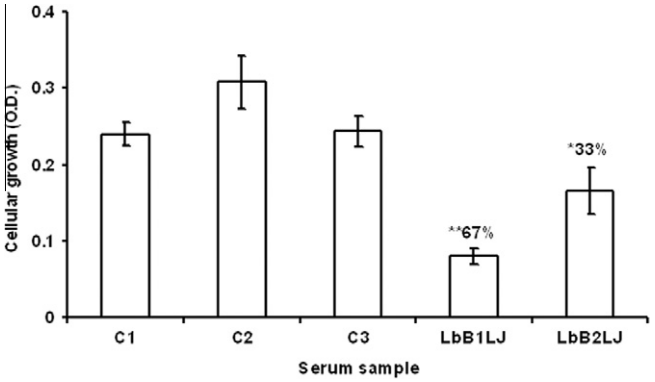

Fig. 3. Antileishmanial activity. The *L. braziliensis* promastigotes were maintained in 96-well microtiter plates at 24 °C and exposed to mouse polyclonal serum anti-LbB1LJ (LbB1LJ) or anti-LbB2LJ (LbB2LJ) at dilution 1:100 for 24 h. Control containing medium alone (C1), serum sample from healthy rabbit (C2; diluted 1:100) or mouse control serum (C3; diluted 1:100) were also included in this assay. The antileishmanial activity was determined by the colorimetric MTT method. The results are expressed as the mean of the optical density ± S.D. of three different experiments performed in triplicate. The statistical significance of difference between mouse control serum (C3, 100% cell growth) and immune serum was determined using Student t test. *P* value is *0.05 or **0.01.

native form and, as predicted by molecular modelling (Faria-Pinto et al., 2008; Maia et al., 2011), its domain B exposed for antibody binding. These new data could to orient site-directed mutagenesis within Domain B from an active recombinant of the *L. braziliensis* NTPDase 1, identifying the amino acid residues that likely interact with the antibodies.

These results stimulate us to test the *L. braziliensis* promastigotes sensitivity to antibody-mediated growth inhibition *in vitro*, and the two immune sera were significantly cytotoxic. The immune serum anti-LbB1LJ that inhibits with more efficiency the NTPDase 1 activity reduces 67%, whereas immune serum anti-LbB2LJ reduces 33% the promastigotes growth, suggesting a direct antibody binding which distinctly inhibits enzyme activity and causes the death of the parasite. A polyclonal immune serum against *Trypanosoma* cruzi NTPDase 1 significantly inhibited the infectivity of trypomastigotes *in vitro* (Santos et al., 2009). In previous studies, the antigenicity of *Toxoplasma gondii* NTPDase isoforms was revealed (Asai et al., 1992), and its catalytic activity was almost totally inhibited by monoclonal antibodies (Kikuchi et al., 2001). Recently was shown that monoclonal antibodies against antigenic *T. gondii* NTPase II inhibited its catalytic activity and significantly reduced the infectivity of tachyzoites (Tan et al., 2010). In addition, a recombinant form of the *T. gondii* NTPase II elicits a strong humoral and cellular immune responses in BALB/c mice inducing partial protection against virulent *T. gondii* strains, and it was indicated as an effective candidate for the development of a vaccine against toxoplasmosis (Tan et al., 2011). Rabbit polyclonal anti-potato apyrase antibodies (Faria-Pinto et al., 2006) or mouse polyclonal antibodies against a synthetic peptide (r175–194) belonging to the domain B from the *Schistosoma mansoni* ATP-Dase 2 (Mendes et al., 2011) did not recognize mammalian NTPDases, corroborated by the lower identity found between domains B from either potato apyrase or parasites and mammalian NTPDases (Faria-Pinto et al., 2008; Maia et al., 2011). We hypothesize that the synthetic peptides LbB1LJ or LbB2LJ, or its derivatives, could induce immune response capable to inhibit invading promastigote forms, and could be tested in experimental protocols of vaccine.

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